Original Article

Single-cell analysis reveals islet autoantigen's immune activation in type 1 diabetes patients

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In this study, we used single-cell sequencing, which can comprehensively detect the type and number of transcripts per cell, to efficiently stimulate peripheral blood mononuclear cells of type 1 diabetic patients with overlapping peptides of GAD, IA-2, and insulin antigens, and performed gene expression analysis by single-cell variable-diversity-joining sequencing and T-cell receptor repertoire analysis. Twenty male patients with type 1 diabetes mellitus participating in the KAMOGAWA-DM cohort were included. Four of them were randomly selected for BD Rhapsody system after reacting peripheral blood mononuclear cells with overlapping peptides of GAD, IA-2, and insulin antigen. Peripheral blood mononuclear cells were clustered into CD8$^+$ T cells, CD4$^+$ T cells, granulocytes, natural killer cells, dendritic cells, monocytes, and B cells based on Seurat analysis. In the insulin group, gene expression of inflammatory cytokines was elevated in cytotoxic CD8$^+$ T cells and Th1 and Th17 cells, and gene expression related to exhaustion was elevated in regulatory T cells. In T cell receptors of various T cells, the T cell receptor β chain was monoclonally increased in the TRBV28/TRBJ2-7 pairs. This study provides insights into the pathogenesis of type 1 diabetes and provides potential targets for the treatment of type 1 diabetes.

***Key Words*:** **type 1 diabetes, single-cell sequence, overlapping peptides, islet autoantigen**

Type 1 diabetes mellitus (T1DM) is a type of autoimmune disease in which the immune system attacks the islet cells responsible for insulin production in the body, reducing or stopping insulin production. From previous research, it has been suggested that both CD4$^+$ T cells and CD8$^+$ T cells are necessary for the development of T1DM, where autoreactive T cells differentiate into effector cells by engaging with islet-associated autoantigens on local antigen-presenting cells.[1,2] CD4$^+$ T cells are responsive to insulin, and CD8$^+$ T cells play a major role in killing β cells. Various other immune cells, including B cells, NK cells, and dendritic cells, have been reported to be involved in the progression of T1DM. CD8$^+$ T cells predominantly infiltrate the islets, but require the assistance of CD4$^+$ T cells for their activation and proliferation. Early models of T cell differentiation focused on the dichotomy between T helper T1 (Th1) and T2 (Th2) types, and T1DM was thought to be primarily a Th1-mediated condition,[3] however, further studies have also reported the involvement of T helper T17 (Th17)[4] and regulatory T cells (Treg).[5]

CD4$^+$ T cells and CD8$^+$ T cells possessing unique T cell receptors (TCRs) that recognize self-islet antigens are activated as single clones.[6,7] CD4$^+$ T cells are responsible for regulating the immune response and providing instructions to other immune cells. In T1DM, CD4$^+$ T cells contribute to disease progression by triggering or coordinating attacks on islet cells.[8,9] Cytotoxic CD8$^+$ T cells play a major role in the destruction of beta cells during the development of T1DM.[10,11] Therefore, elucidating the diversity of TCRs in CD4$^+$ T cells and CD8$^+$ T cells is vital for understanding the pathogenesis of T1DM. TCR antigen-binding sites are determined by gene rearrangement, resulting in a diversity of 10^{10} possibilities. Previously, revealing the complete repertoire of these vast antigen receptors was challenging. However, the advancement of next-generation sequencing technology now enables the identification of TCR gene sequences expressed by targeted cell populations at the individual clone level. Currently, comprehensive immune sequencing techniques are applied to monitor *in vivo* immune responses, develop antibody therapeutics, vaccines, and cellular medicines, holding the potential for significant innovations across various medical fields. Comprehensive immune sequencing is utilized in the analysis of conditions such as T1DM, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and more.[12] We have analyzed the pairing of α and β chains constituting TCRs in peripheral blood mononuclear cells (PBMCs) from patients with T1DM at the single-cell level, identifying genes whose expression increases not only in T cells but also in TRA and TRB rearrangements.[13] T1DM develops when effector T cells, induced by viral or bacterial infections, cross-react with autoantigens and attack their own pancreatic beta cells.[14] Although autoreactive T cells are thought to be central to the pathogenesis of the disease, there have been reports on numerical abnormalities or lack thereof, and qualitative abnormalities have recently attracted attention.[15] Therefore, exhaustion of effector T cells is thought to be effective against T1DM, an autoimmune disease. A therapeutic effect has been confirmed by CD3 monoclonal antibody-induced effector T cell exhaustion in the treatment of T1DM.[16,17] In particular, a clinical trial with teplizumab, a CD3 monoclonal antibody, successfully prevented the onset of T1DM in high-risk groups.[18] However, its effectiveness remains limited, and identification of its TCR sequence is needed to identify individuals at a high risk of developing T1DM more accurately and to develop new therapies that specifically eliminate autoreactive T cells. Cole *et al.*[6] identified the 1E6 TCR, which has a binding site called "hot spot" and structural evidence that once the core portion of the 3-amino acid peptide binds strongly, the remainder of the peptide can react with any sequence. The structure of the TCR allows for flexibility in antigen specificity, and although it has an inherent affinity for foreign antigens, it can also react with insulin peptides and pancreatic beta cell component peptides, even though they have low affinity.

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Recent studies on SARS-CoV-2 have focused on the use of overlapping peptides, which are designed to overlap each peptide with 11 amino acid residues back and forth in a peptide pool consisting of 15 mer.[19,20] These peptides cover the entire antigenic protein of interest and stimulate $CD4^+$ T cells in a highly efficient and antigen-specific manner. Patients with T1DM produce autoantibodies such as the 65 kDa isoform of glutamic acid decarboxylase (GAD65), tyrosine phosphatase-related islet antigen 2 (IA2), and insulin antibodies. GAD65 is expressed on pancreatic beta cells and is considered an important autoantigen in the development and pathogenesis of T1DM.[21] Islet antigen 2 (IA-2), a member of the protein tyrosine phosphatase family, is considered one of the major autoantigens in T1DM.[22] However, the exact role of IA-2 antigen in the etiology of T1DM remains unknown. Further, insulin antigen is a candidate autoantigen for T1DM because it is the only known β-cell specific antigen associated with T1DM. Previous reports have detected $CD4^+$ and $CD8^+$ T cells recognizing human hybrid insulin peptides[23] and other post-translationally modified islet antigens,[24] but no studies have yet analyzed their TCR sequences or other characteristics.

This research aimed to elucidate the causes of T1DM and develop novel prevention methods by stimulating peripheral blood mononuclear cells (PBMCs) with islet-related autoantigens and using single-cell sequencing to examine changes in gene expression primarily in T cells and the VDJ rearrangement of T cell receptors. The following experiments were conducted. The following articles are based on STREGA's reporting checklist.

Materials and Methods

Study design and participants. The KAMOGAWA-DM cohort study is an ongoing prospective cohort study approved by the Ethics Committee of Kyoto Prefectural University of Medicine since 2013 (Kyoto, Japan, RBMR-E-466).[25] Informed consent was obtained from all patients participating in the KAMOGAWA-DM cohort study. The study was conducted in accordance with the Declaration of Helsinki. Between April and May 2021, a random selection was made from T1DM patients who visited our diabetes outpatient clinic at our institution. PBMCs were collected on the day of the visit, and the experiments were conducted on the same day without freezing and storing the samples. Among the 24 patients, none of them developed any apparent infections during the study period. The diagnosis of T1DM was based on the criteria of the American Diabetes Association,[26] and patients with Type 1A diabetes (i.e., immune-mediated) were chosen following the recommendations of the American Diabetes Association Expert Committee.[27]

Isolation of PBMCs and *in vitro* culture of PBMCs. First, approximately 10 ml of peripheral blood was collected in CPT tubes (BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube, BD BioSciences, San Jose, CA) and PBMCs were extracted as per manufacturer's instructions. Following this, 1.5×10^6 PBMCs from each group were seeded in a 96-well plate using RPMI containing 5% human AB serum. Next, the negative control (NC) group was cultured with sterile PBS, the insulin antibody group was cultured with PepTivator® Insulin, the IA-2 antibody group was cultured with PepTivator® IA-2, and was the anti-GAD antibody group was cultured with PepTivator® GAD65 in separate wells at a concentration of 0.6 nmol/ml according to manufacturer's instructions, and the plates were incubated at 37°C in a CO_2 incubator for 2 h. The reason for the 2-h setting is to avoid contamination from nonspecific cells, which make up the majority of cells in each culture well, because nonspecific cell reactions take longer than antigen-specific reactions.

Single-cell sequencing and data assessment. One previous report suggested that the BD system may be more suitable than 10x scRNASeq for inaccessible patient samples.[28] The BD system was employed in this study because cell death due to antigen stimulation was anticipated. PBMCs were not pre-sorted and whole PBMCs were used for single cell sequencing analysis. Cells were labeled with a specific sample tag using the BD Rhapsody Human single-cell multiplexing kit and AbSeq antibodies against major human immune markers (BD Rhapsody Immune Response Panel, comprising 399 genes, catalog number 633750). This labeling process was carried out for 30 min on ice. Following thorough washing, cells with different sample tags were combined in equal proportions, and a maximum of 20,000 cells were loaded onto a BD Rhapsody Cartridge.

Single-cell capture and cDNA library preparation were performed using the BD Rhapsody Express Single-Cell Analysis System by BD Biosciences, following the manufacturer's instructions. Libraries were prepared for single-cell transcriptomes targeting immune profiles (TTA), whole transcriptomes (WTA), Ab-tagged index sequences targeting 30 immune cell-associated surface antigens, and multiple sample tags using BD Rhapsody TTA and WTA amplification kits, as per the manufacturer's guidelines.

For the mRNA-targeted library, the BD Rhapsody™ immune response panel was utilized, which included primer pairs targeting 397 genes commonly expressed in human immune cells. Sequencing was conducted on an Illumina HiSeq 6000 platform (Novogene, China). The resulting FASTQ sequencing files were analyzed using the BD Rhapsody Analysis Pipeline ver. 1.10.1 to generate an expression matrix.

The FASTQ files derived from the sequencing data were processed using the BD Rhapsody Targeted Analysis Pipeline with V(D)J processing, a software tool provided by BD Biosciences, on the Seven Bridges Platform (https://www.sevenbridges.com/d). First, low-quality read pairs were filtered out based on criteria such as read length, average base quality score, and highest single-base frequency. Subsequently, the high-quality R1 reads were analyzed to identify cell labels and unique molecular identifier (UMI) sequences. The high-quality R2 reads were aligned to reference panel sequences (specifically, the Human T cell Expression panel, Supplemental Table 1*) and TCR gene segments sourced from the International ImMunoGeneTics Information System (IMGT.org) using the Bowtie2 program. CDR3 regions were determined using IGBLAST. Reads with identical cell labels, UMI sequences, and genes were collapsed into single molecules. The obtained molecule counts underwent error correction using algorithms developed by BD Biosciences, including recursive substitution error correction and distribution-based error correction (DBEC). The DBEC-adjusted molecule count data, generated by the Rhapsody pipeline, were imported into SeqGeq ver. 1.6.0. Subsequently, quality control steps were implemented to filter out cells that were significantly smaller and had low expression levels of genes (termed as "dead cells"). Following the quality control phase, dimensional reduction and unbiased clustering were performed within SeqGeq using the Seurat plug-in. Specifically, Seurat was configured to include all genes used, incorporating QC functions, log normalization, and the utilization of the uniform manifold approximation and projection (UMAP) technique for dimensionality reduction. These plugins generated various data outputs, including UMAP representations, lists of genes that were upregulated and downregulated, and annotation information, all utilizing the PBMC gene model. UMAPs for the first four patients were plotted. Canonical Correlation Analysis (CCA) was performed to reduce batch effects, and UMAP analysis was performed. UMAP was then plotted by the four antigens. To identify cells in detected clusters, cell populations were automatically identified using the ScType package. The ScType platform has been developed as an open-source, interactive web tool accessible at https://sctype.app. This platform seamlessly interfaces with the ScType marker database and offers rapid and entirely automated cell type anno-

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Table 1. Subjects profile

	Type	Age	Sex	Insulin (U/ml)	IA-2 (U/ml)	GAD (U/ml)
Sample 1	IA	48	Male	6.7	<0.4	12.5
Sample 2	IA	50	Male	12.1	<0.4	18.1
Sample 3	IA	49	Male	15	<0.4	15
Sample 4	IA	52	Male	7.7	<0.4	23.7

tation, catering to various biomedical applications. Incorporating cluster information and TCR CDR3 data for each individual cell was achieved through the utilization of the VDJExploler plug-in within SeqGeq. To assess the structural diversity of genes, the Shannon index H was computed as a parameter.[29]

$$H' = -\sum_{i=1}^{S} p_i ln p_i$$

S: Number of genes observed in the sample
pi: Ratio of genes *i* to the total sample

Detection of T cells using whole transcriptome single-cell RNA sequencing (scRNA-seq) data. Gene expression was evaluated utilizing two publicly accessible genomic datasets that combine information from three mRNA and surface protein expression datasets. A 10k PBMC dataset was created using v3 chemistry, which yielded 7,865 cells that passed quality control. The average number of reads per cell for the mRNA libraries in this 10k PBMC dataset was 35,433. Additionally, a 5k PBMC dataset was generated using NextGEM chemistry, resulting in 5,527 cells that passed quality control. The average number of reads per cell for the mRNA libraries in this 5k PBMC dataset was 30,853. (Source: https://support.10xgenomics.com/single-cell-gene-expression/dataset).

TCR CDR3 motif identification. All TCR CDR3 amino acid sequences from the current study were aligned using the MEME tool from the MEME suite (https://meme-suite.org/meme/tools/meme).[30]

Flowcytometry. Stained cells were analyzed using a fluorescence-activated cell sorting (FACS) Canto II, and data were analyzed using FlowJo ver. 10 software (TreeStar, Ashland, OR). The following antibodies were used for gating of T cells: FITC-CD45RA [561216; clone: 5H9 (RUO); 1/100; BD Pharmingen™, San Diego, CA], PE-CCR7 [552176; clone: 3D12 (RUO); 1/100; BD Pharmingen™], PerCP-CD4 (317432; clone: OKT4; 1/100; BioLegend, San Diego, CA), PerCP/cy5.5-CD8 (344714; clone: SK1; 1/100; BioLegend), APC-CD38 (356606; clone: HB-7; 1/100; BioLegend), APC/Cyanine7-Fixable Viability Dye eFluor™ 780 [Live & Dead (L/D)] (65086514; 1/1,000; eBioscience, San Diego, CA), V450-CD3 [560365; clone: UCHT1 (RUO); 1/100; BD Horizon™, San Diego, CA], V500-HLA-DR (559866; clone: G46-6 (RUO); 1/100; BD Pharmingen™).

Statistical analysis. Data were analyzed and figures were constructed using R (ver. 4.3.1) and GraphPad Prism software (ver. 9.3.1; San Diego, CA). Comparison of the two groups was performed using Welch's *t* test. Comparison of the four groups was performed using one-way analysis of variance with Tukey honestly significant difference test. Statistical significance was set at $p<0.05$.

Results

Study design and analysis of single immune cell profiling in patients with T1DM. To map the immune microenvironment of four T1DM patients (Table 1), we integrated scRNA-seq and single-cell paired TCR analysis from a total of four T1DM patients. In addition, the stimulation of immune cells in the peripheral blood by overlapping peptides of islet autoantigen was used to simulate the early state of T1DM onset (Fig. 1A). All cells were classified into 20 major clusters and annotated with canonical marker gene expression by UMAP (Fig. 1B and Supplemental Fig. 1*), and top 5 genes in each cluster were shown in violin plots (Supplemental Fig. 2*). First, we manually clustered the cells by referring to the genes of immune cells in The Human Protein Atlas (https://www.proteinatlas.org/humanproteome/immune+cell) (Fig. 1C). Cluster 0, 8, 11, 12, and 13 by UMAP were classified into natural killer cells, cluster 1 and 6 by UMAP were classified into monocytes, cluster 2, 5, 9, and 10 by UMAP were classified into $CD8^+$ T cells, cluster 3, 17, and 19 by UMAP were classified into granulocytes, cluster 4, 14, and 15 by UMAP was classified into $CD4^+$ T cells, cluster 7 and 16 by UMAP was classified into B cells, and cluster 18 by UMAP was classified into dendritic cells. Natural killer cells showed predominant expression of *GNLY*, *GZMB*, and *PRF1*.[31,32] Monocytes showed predominant expression of *CXCL8*.[33] *HLA-DPA1* and *HLA-DPB*1 were strongly expressed in dendritic cells and B cells (Fig. 1D). There was no significant difference in the proportion of immune cells present in each group (Fig. 1E). The expression of genes related with cytotoxic, cytokine, CD3/CD4/CD8/TCR, activation, HLA, resting, exhaust, and effector in whole PBMC in Supplemental Fig. 3*.

scRNA-seq reveals $CD8^+$ T cells phenotype. From the immune cells classified in the previous chapter, $CD8^+$ T cells were first extracted and clustered. Seven clusters were then generated (Fig. 2A), and the Top 10 expressed genes in each cluster are shown in the heatmap (Fig. 2B). Among them, cluster 1 was considered as cytotoxic $CD8^+$ T cells due to the elevated expression of *IL2*, *IFNG*, and *TNF*.[34] Moreover, comparison of gene expression in cytotoxic T cells for cytotoxicity and cytokines between each antigen group showed that gene expression of *GZMB*, *TNF*, and *IFNG* was elevated in the insulin group, compared to NC group (Fig. 2C). When cells positive for *TNF* and *IFNG* were plotted in UMAP, most cells were plotted in cluster 1, with more positive cells in the insulin group. On the other hand, *GZMB* was expressed evenly not only in cluster 1 but also in other clusters. (Fig. 2D).

scRNA-seq reveals $CD4^+$ T cells phenotype. Next, we have investigated $CD4^+$ T cells phenotype. Nine clusters were then generated (Fig. 3A), and the Top 10 expressed genes in each cluster are shown in the heatmap (Fig. 3B). Among them, Cluster 0 was considered as naïve Th cells (Th0), cluster 1 as Type 1 T helper (Th1), cluster 4 as Type 2 T helper (Th2), cluster 3 as Type17 T helper (Th17), cluster 2 as activated Th0, cluster 5 and 6 as cytotoxic CD4 T^+ cells and cluster 7 and 8 as Treg. In addition, gene expression in each cell cluster was compared in each antigen group. In Th1, *IL6* and *IFNG* were upregulated in the GAD group, *IL2* in the IA-2 group, and *IL2*, *IL6*, and *IFNG* in insulin group, compared to NC group. In Th17, the expression of *IL12A* was upregulated in IA-2 and Insulin groups, and that of *TNF* was upregulated in Insulin group. Because HLA-related genes were strongly expressed in activated Th0, their expression was compared among the four groups. The expression of *HLA-A*, *HLA-DPB1*, and *HLA-DRA* was upregulated in the insulin group

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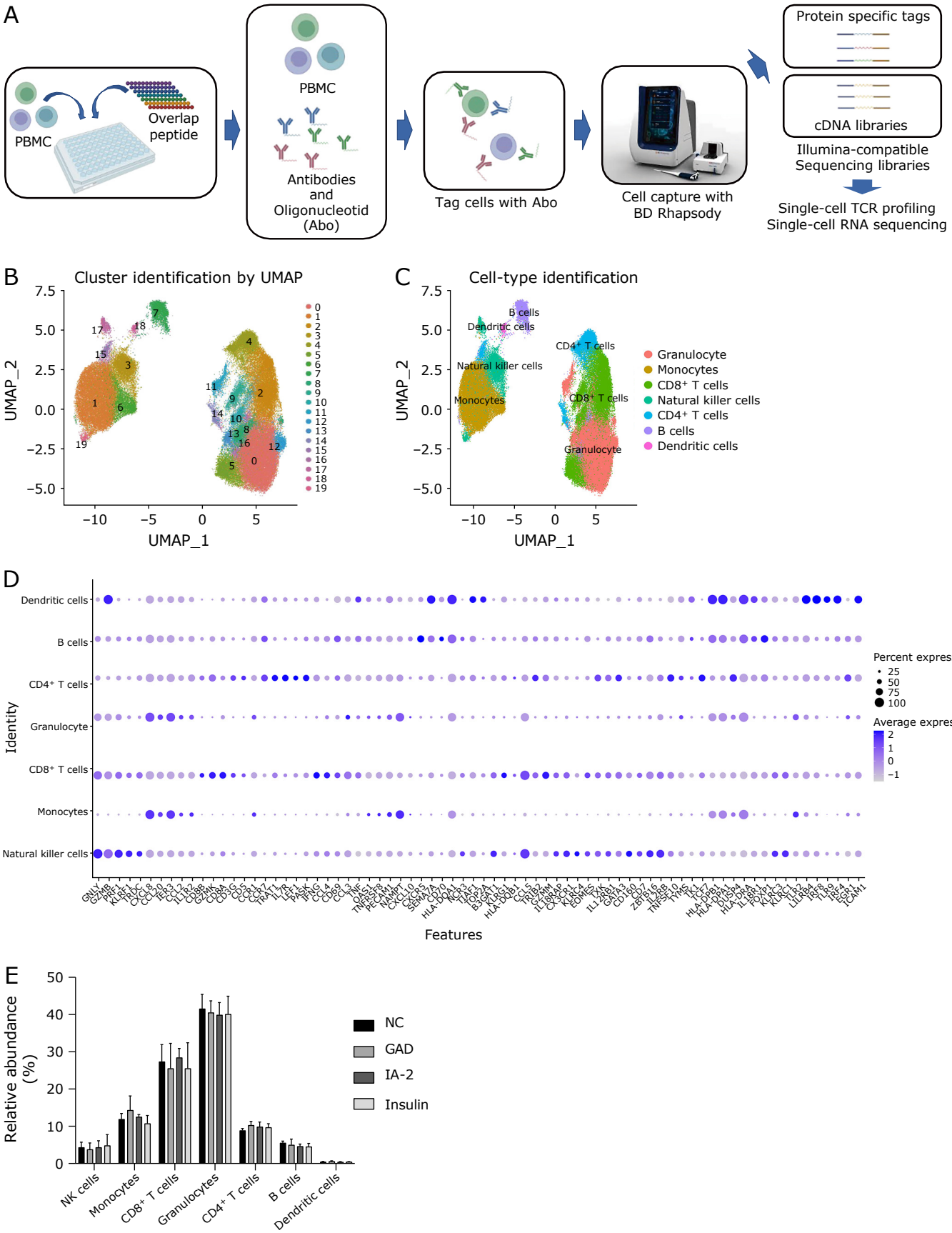

Fig. 1. Cluster identification of PBMC in type 1 diabetic patients. (A) Scheme of experimental study design. (B) Cluster identification by UMAP. (C) Cell type identification by manual referring to the genes of immune cells in The Human Protein Atlas. (D) Dot plots for the expression of the top 5 marker genes in each cell cluster. (E) Relative abundance of each immune cells by antigen. NK, natural killer.

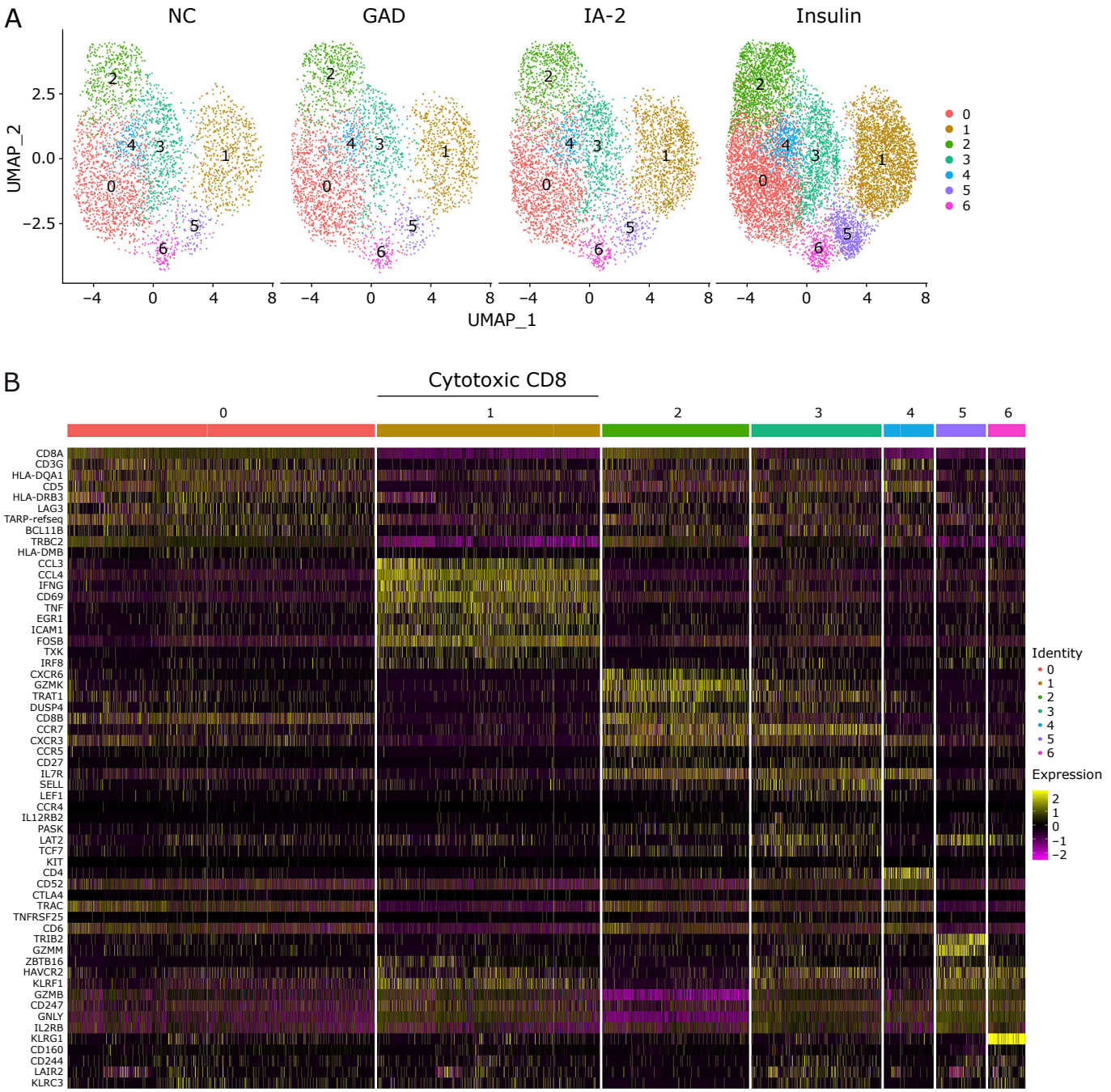

Fig. 2. Single cell RNA sequencing reveals CD8⁺ T cells phenotype. (A) UMAP analysis of CD8⁺ T cells. (B) Single cell resolution heatmap analysis of top 10 genes for each identified CD8⁺ T cells subset. (C) Violin plots of normalized expression for selected markers related with cytotoxic and cytokine mapped across the CD8⁺ T cells cells subsets of cluster 1. (D) Feature plots of *GZMB*, *TNF*, and *IFNG*. Data were analyzed using one-way analysis of variance (ANOVA) with Holm-Šídák's multiple comparison test. *p<0.05.

compared to the NC group. CD9 has been reported to contribute to cell activation among Th0.[35] In Th2, *CCR3*, and *IL4* were upregulated in the GAD group and *IL4* and *IL13* in the insulin group, compared to NC group. In cytotoxic CD4⁺ T cells, the expression of *GZMA*, *GZMB*, and *GZMH* in the insulin group was higher than that in the NC group. Finally, in Treg, the expression of genes related with exhaust was investigated. The expression of *CD244* and *TGFB1* were upregulated in the GAD group, *PDCD1*, *TIGIT*, and *TGFB1* in the IA-2 group, and CD244, CD160, and TGFB1 in the insulin group, compared to NC group (Fig. 3C).

Multicolor flow cytometry analyses of CD8⁺ T cells and CD4⁺ T cells. Multicolor flow cytometry was used to demonstrate whether cytokine production upon stimulation with the insulin antigen was more advanced than with the other two antigens (Fig. 4A and Supplemental Fig. 4*). The ratio of CD8⁺ T cells in CD4⁺ T cells were not different between the four groups (Fig. 4B). On the other hand, the frequency of IFNG positive cells in CD8⁺ T cells and CD4⁺ T cells of insulin group were higher than NC, GAD, and IA-2 groups, which was also true for TNF-positive cells (Fig. 4C and D).

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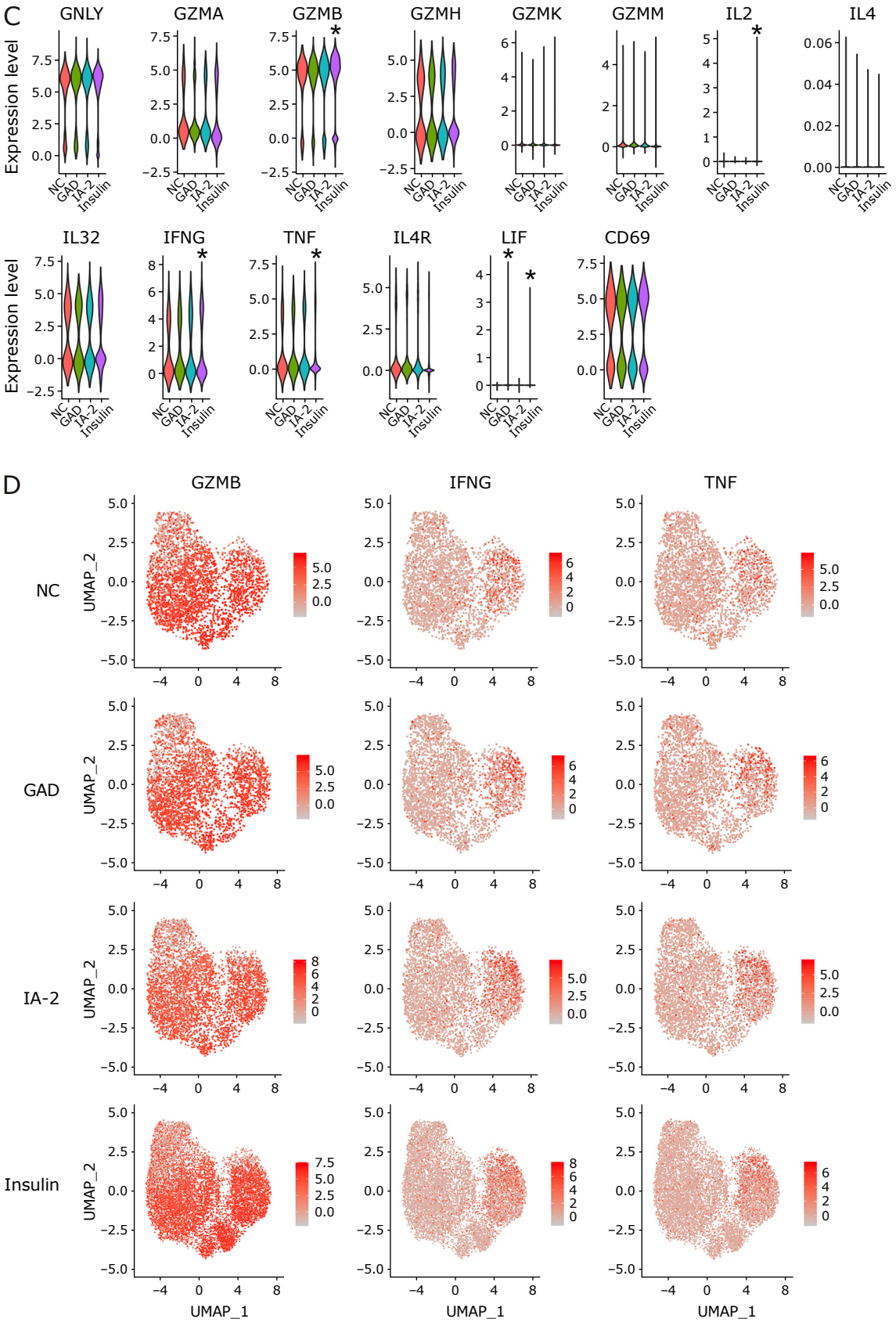

Fig. 2. Continued

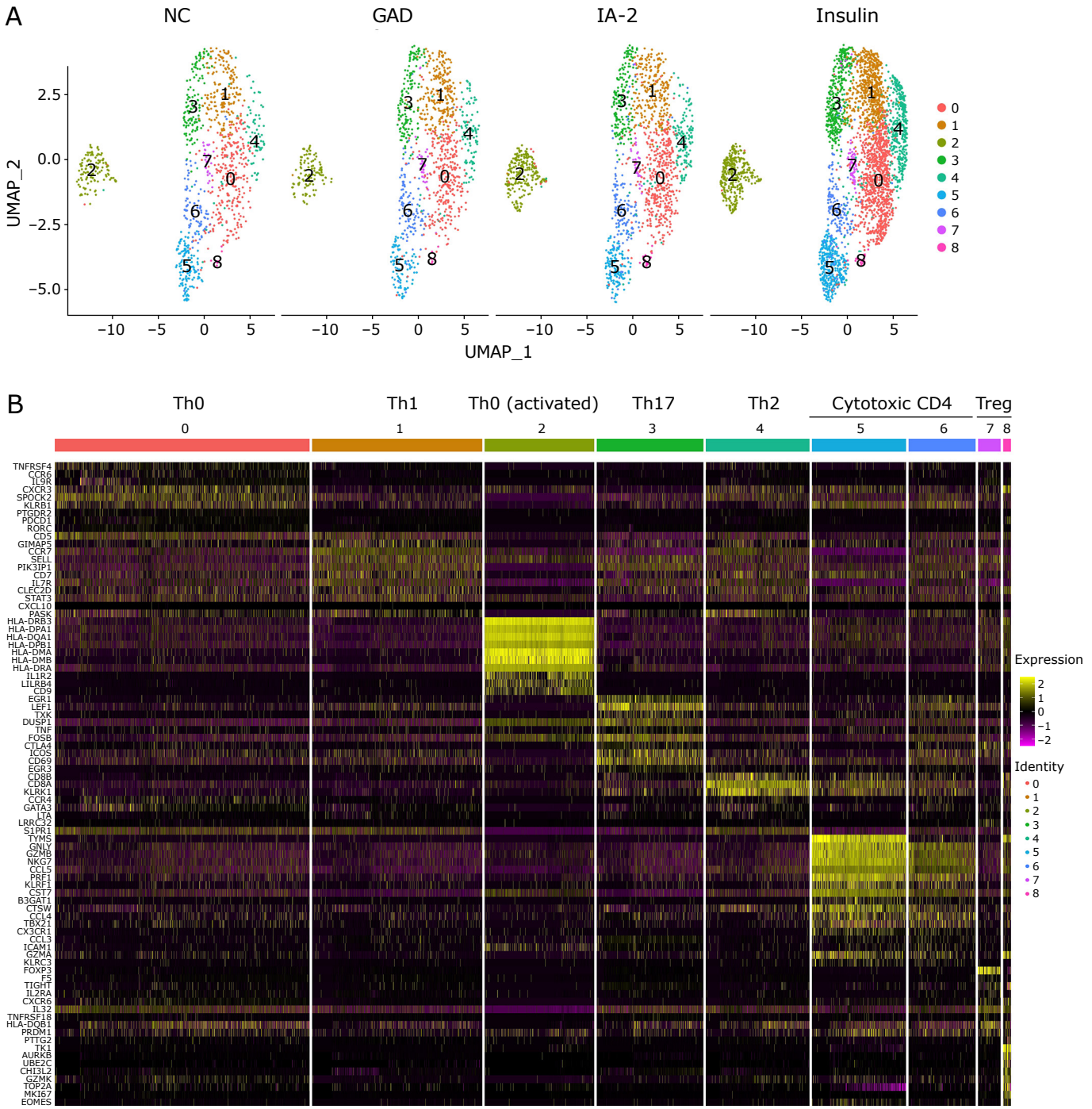

Fig. 3. Single cell RNA sequencing reveals CD4⁺ T cells phenotype. (A) UMAP analysis of CD4⁺ T cells. (B) Single cell resolution heatmap analysis of top 10 genes for each identified CD4⁺ T cells subset. (C) Violin plots of normalized expression for selected markers mapped across the CD4⁺ T cells subsets. Data were analyzed using one-way ANOVA with Holm-Šídák's multiple comparison test. *p<0.05.

scRNA-seq reveals phenotypes of NK cells, dendritic cells, and B cells. The same analysis was performed for NK cells, dendritic cells, and B cells as for CD8⁺ T cells and CD4⁺ T cells. NK cells were divided into four clusters (Fig. 5A), with cluster 1 having elevated expression of cytokines such as *IL32* and HLA-related genes (Fig. 5B). Expression of HLA-DR on NK cells has been reported to stimulate activation and proliferation of certain T cells.[36] Thus, the expression of cytotoxic-related genes, HLA-related genes, and cytokine-related genes were compared among the four groups in HLA-DR-positive NK cells (Fig. 5C). The expression of *GZMH*, *HLA-DQA1*, *IL6*, and *IL32* in the insulin group was higher than that in the NC group.

Dendritic cells were classified into three clusters (Supplemental Fig. 5A*); GNLY has been reported to be able to recruit and activate antigen-presenting cells and to promote antigen-specific immune responses.[37] The expression of *GNLY* was upregulated in cluster 1 (Supplemental Fig. 5B*), while the expression of *GNLY*, *GZMA*, *GZMB*, *GZMH*, and *GZMK* was upregulated in the insulin group (Supplemental Fig. 5C*).

B cells were classified into eight groups (Supplemental Fig.

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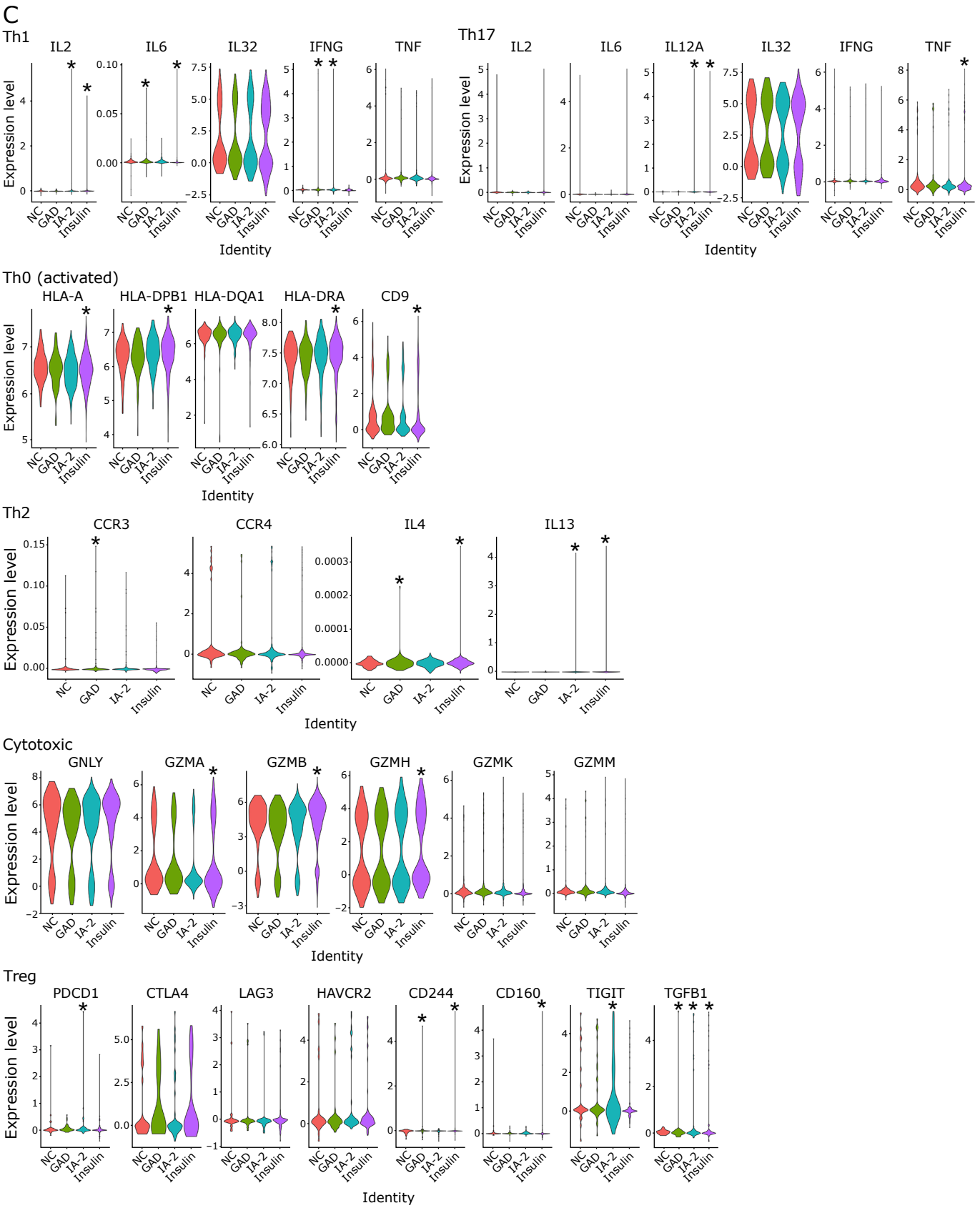

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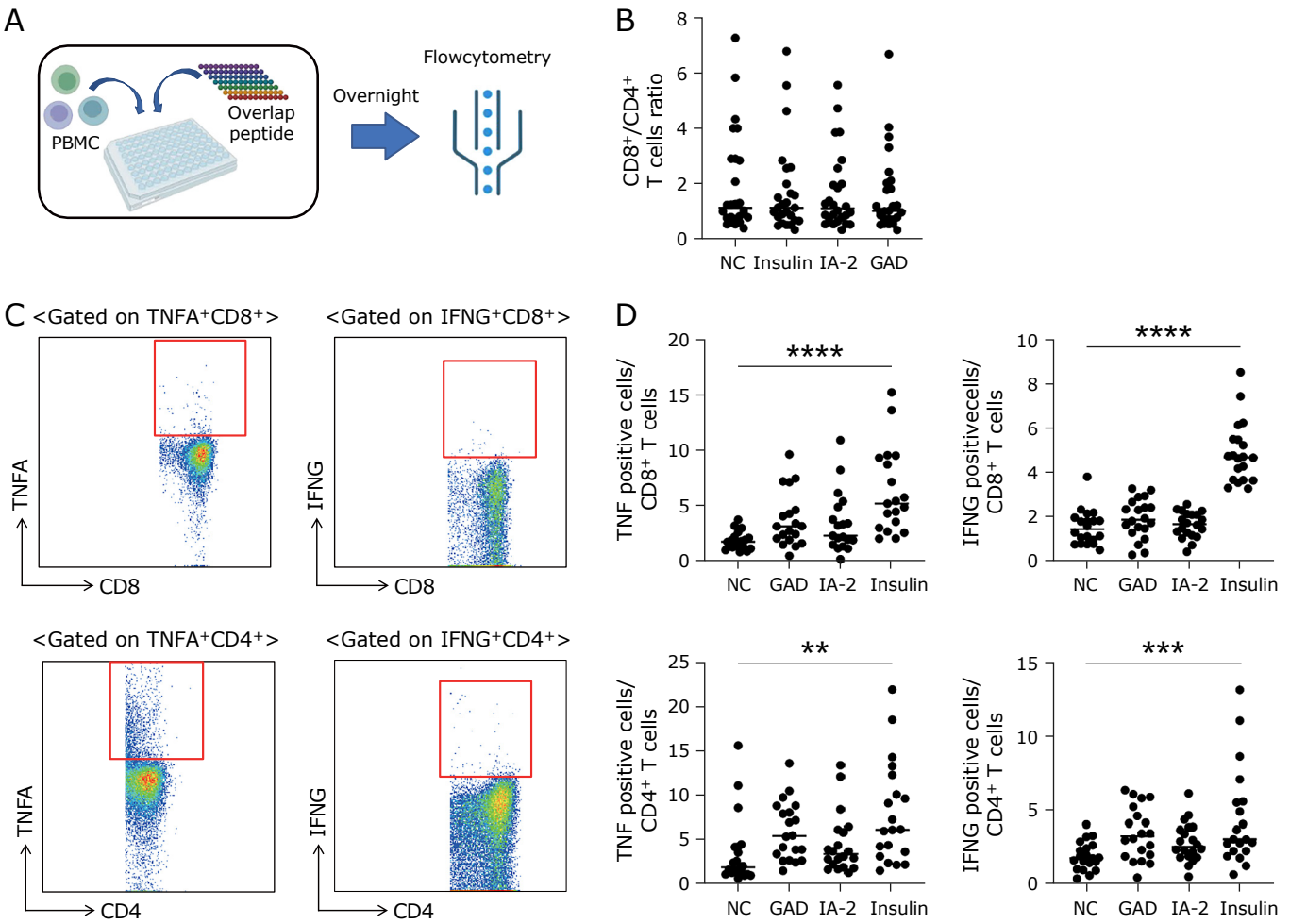

Fig. 4. Flow cytometric analysis of T cells from type 1 diabetic patients treated with antigen. (A) Scheme of experimental study design. (B) Ratio of CD8⁺ T cells/CD4⁺ T cells. (C) Gating strategy of TNFA⁺CD8⁺, IFNG⁺CD8⁺, TNFA⁺CD4⁺, IFNG⁺CD4⁺ T cells. (D) Percentage of TNFA⁺ and IFNG⁺ cells in CD8 or CD4⁺ T cells by antigen. Data are presented as mean ± SD values. Data were analyzed using one-way ANOVA with Holm-Šídák's multiple comparison test. **p<0.01, ***p<0.001, ****p<0.0001.

6A*). Elevated HLA-DR expression on B cells has been reported to allow efficient presentation of low-affinity peptides to T cells.[38] In Cluster 0, which *HLA-DR* was strongly expressed, *IL6*, *IFNG*, and *TNF* in the IA2 group and *TNF* and *TGFB1* in the insulin group Expression was elevated, compared to the NC group (Supplemental Fig. 6B and C*).

Expanded TCR clones and selective usage of V(D)J recombination by antigen. We extracted each of the TCR genes that mapped to the cell population of each cluster to reveal the TCR repertoire of each cluster. The TCR repertoires of all cells, cytotoxic CD8⁺ T cells, cytotoxic CD4⁺ T cells, Th1, Th2, Th17, and Treg are shown in the bubble plot (Fig. 6A). In all cells, TRAV1-2/TRAJ33 and TRAV29/DV5/TRAJ42 pairs were frequently observed in TRA, and TRBV28/TRBJ2-7 pairs in TRB. A similar trend was observed in Cluster 2. In cluster 5, on the other hand, no clearly expanded clones were observed in TRA, but TRBV27/TRBJ2-7 was increased in TRB as in the other two clusters. The Shannon index of the TCR repertoire in the insulin group was significantly lower than in the NC, IA-2, and GAD groups (Fig. 6B); CDR3 length diversity in the insulin group was higher than in the NC group.

Comprehensive motif analysis of TCR. The motif-based sequence analysis tool MEME was used to identify consensus amino acids in grouped CDR3 sequences. Motif analysis common to GAD, IA-2, and insulin groups was performed in cytotoxic CD8⁺ T cells, cytotoxic CD4⁺ T cells, Th1, Th2, Th17, and Treg, respectively. The amino acid sequence of TRA showed some similarities but different sequences among the immune cells. Among them, cytotoxic CD8⁺ T cells and Th17 had similar sequences. On the other hand, the sequence of TRB was similar in all groups (Fig. 6C).

Discussion

In this study, we quantitatively and qualitatively evaluated changes in immune status using PBMCs of patients with T1DM and typical islet-associated autoantigen such as insulin, IA-2, and GAD65. Interestingly, there was no obvious difference in the ratio of each immune cell, such as CD8⁺ T cells, CD4⁺ T cells, NK cells, B cells, and dendritic cells, stimulated by these antigens between the groups, but there were clear differences in the gene expression of each immune cell.

Studies with NOD mice have shown that the onset of T1DM is dependent on both CD4⁺ and CD8⁺ T cells as well as dendritic cells and NK cells.[39,40] CD3-specific antibodies that induce T cell tolerance reversed the onset of T1D in NOD mice, highlighting the important role of T cells in sustained β-cell destruction.[41] T cell-mediated β-cell death can occur in several ways. CD8⁺ T cells can kill pancreatic β-cells through MHC class I-mediated cytotoxicity, and cytokines such as IFN-γ secreted by

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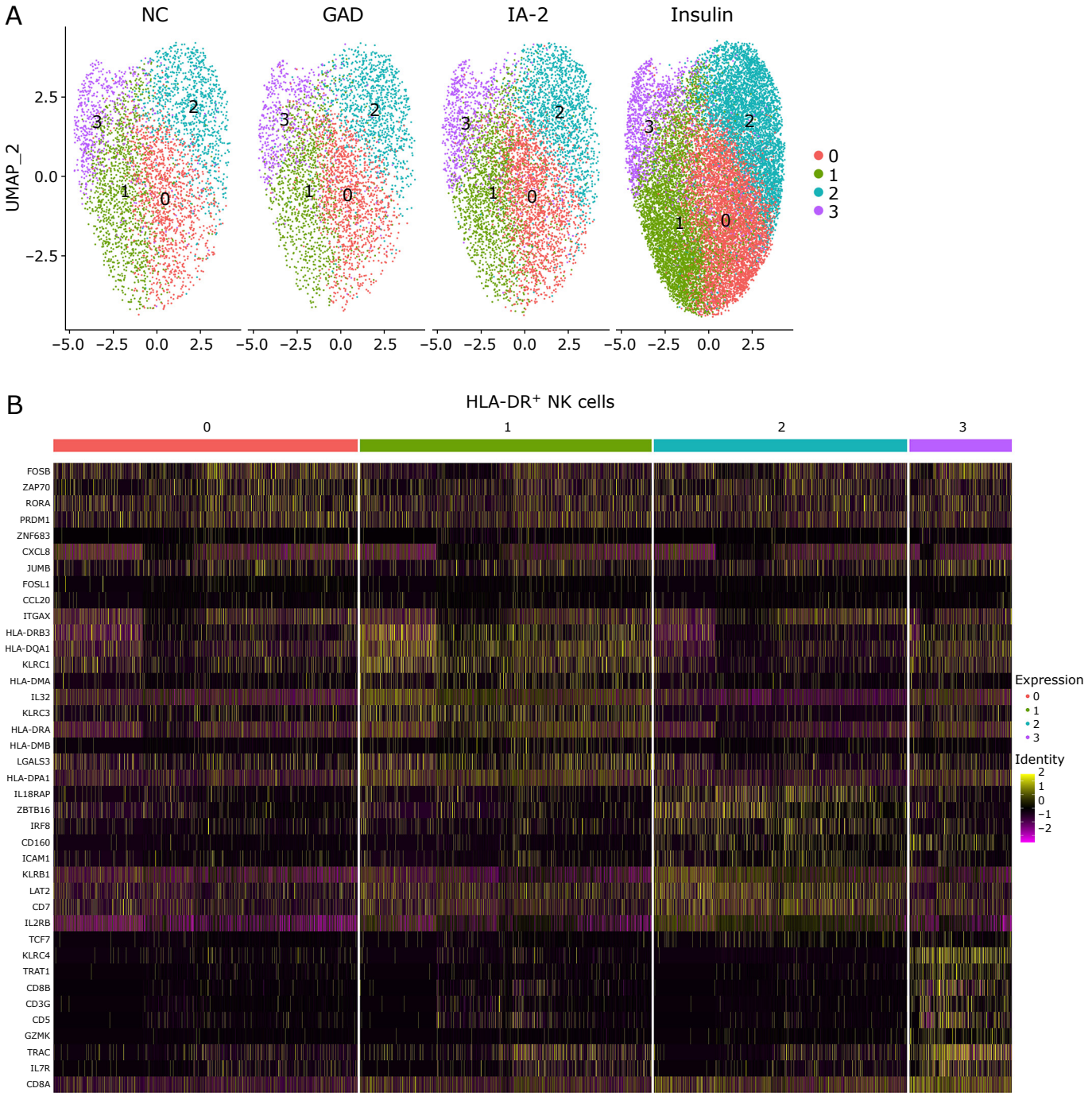

Fig. 5. Single cell RNA sequencing reveals NK cells phenotype. (A) UMAP analysis of NK cells. (B) Single cell resolution heatmap analysis of top 10 genes for each identified NK cells subset. (C) Violin plots of normalized expression for selected markers mapped across the NK cells of cluster1 subsets. Data were analyzed using one-way ANOVA with Holm-Šídák's multiple comparison test. *p<0.05.

CD8$^+$ T cells, CD4$^+$ T cells, dendritic cells, and NK cells, which in turn induce β-cell expression of the death receptor FAS and activation of FAS by activated T cells expressing FAS ligands may induce β-cell apoptosis. Chemokine production by β-cells leads to further recruitment of mononuclear cells to the affected area, thereby enhancing inflammation.[42] In addition, IFN-γ activates macrophages and induces increased production of inflammatory cytokines such as IL1β and TNF. β cells express high levels of IL-1 receptors and appear to be more sensitive to IL-1β-induced apoptosis than other endocrine cells in the islets; this crosstalk between T cells and macrophages undoubtedly exacerbates immune-mediated stress on beta cells and contributes to their destruction. Furthermore, the balance between Th1 and Th2 responses has also been implicated in the development of T1DM: analysis of T cells in PBMC from patients with T1DM supported an IFN-γ-dominant response to islet autoantigen and revealed that the balance between IFN-γ and IL-10 was different between patients and healthy controls.[43] Furthermore, the association of Th17 cells with Th1 is also essential in the pathogenetic basis of T1DM. As mentioned earlier, Th1 and IFN-γ are factors in the pathogenesis of T1DM, and it has been reported that double knockout mice for IL-17 and IFN-γ receptors have a significantly

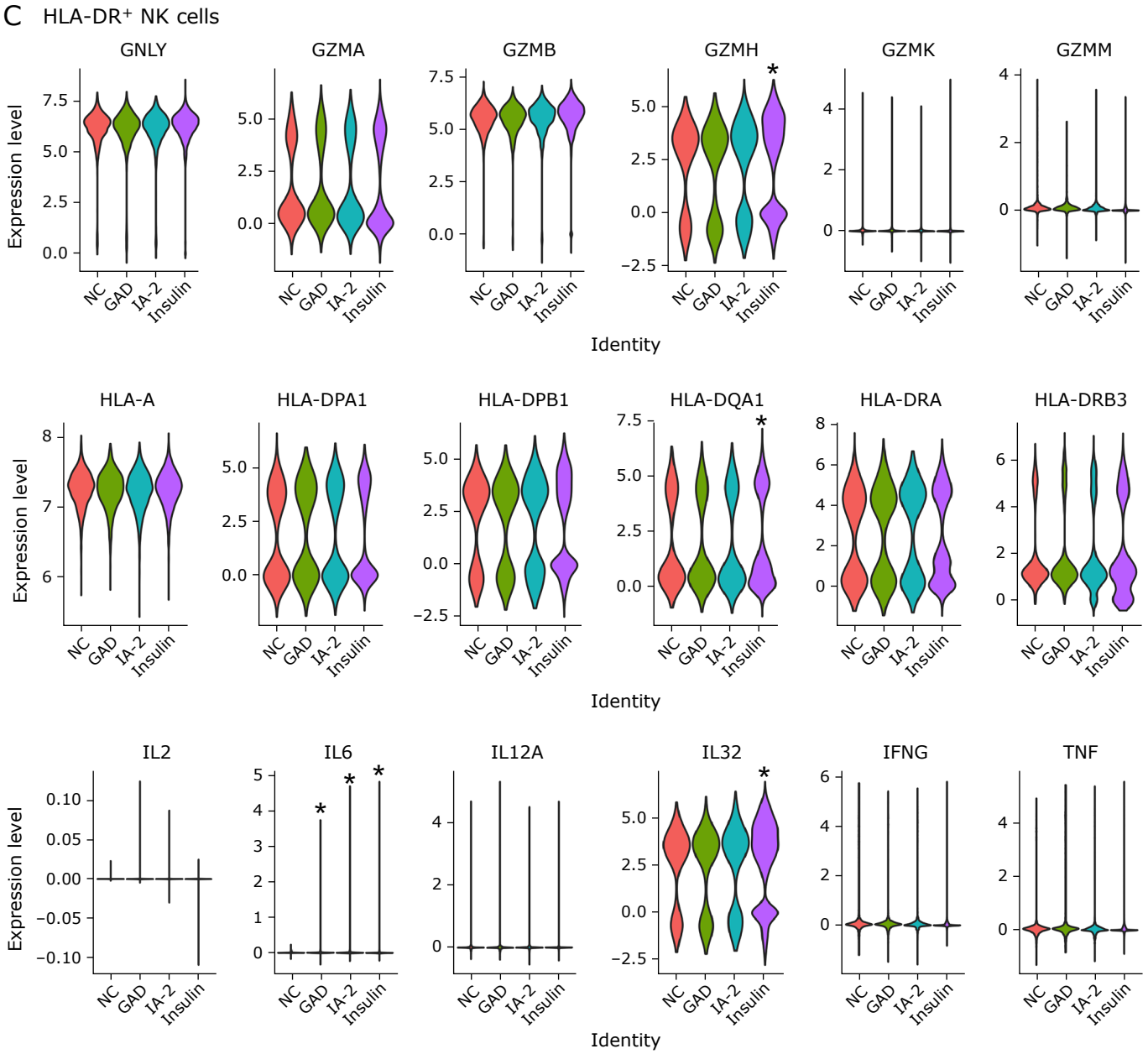

Fig. 5. Continued

delayed onset of diabetes compared to IL-17 single knockout mice.[44] This suggests that Th17 cells may cooperate with Th1 and IFN-γ to mediate inflammation in diabetes. On the other hand, Li *et al.*[45] have shown a novel mechanism for Th17-mediated diabetes that is independent of IFN-γ but dependent on TNF. While T cells play a pathological role in the development of T1DM, there is also evidence to support a role for T cells in preventing β-cell destruction. CD28-deficient NOD mice lacking Treg develop T1DM at an accelerated rate,[46] and patients with mutations in Treg might develop T1DM[47] and this underscores the importance of Tregs in regulating the development of this autoimmune disease. Numerous reports support the importance of T cells in the pathogenesis of T1DM, while other data suggest the involvement of other cell types, such as B cells, which may serve as antigen-presenting cells that maintain islet antigen-specific T cell activity. In NOD mice, depletion of B cells by gene targeting or antibody therapy has been shown to suppress T1DM development.[48,49] The results suggest that B cells may play a role as antigen-presenting cells that maintain islet antigen-specific T cell activity.[48,50]

As mentioned above, many immune cells have been implicated in the development of T1DM. In this study, we found characteristic changes in gene expression in each immune cell after exposure to islet-associated autoantigens compared to the NC group. In the insulin group, *IFNG* expression was increased in cytotoxic $CD8^+$ T cells and Th1, and *TNF* expression was increased in Th17. Moreover, the expression of inhibitory receptors such as *CD244* and *CD160* was increased in Tregs in the insulin group.[51] We examined gene expression in clusters of NK cells that characteristically express high levels of *HLA-DR* genes; HLA-DR molecules are involved in antigen presentation and this molecule is highly expressed on APCs; HLA-DR expression in NK cells has been shown to increase IFNγ production,[52,53] high proliferative activity,[54] and degranulation rate[55] as well as the

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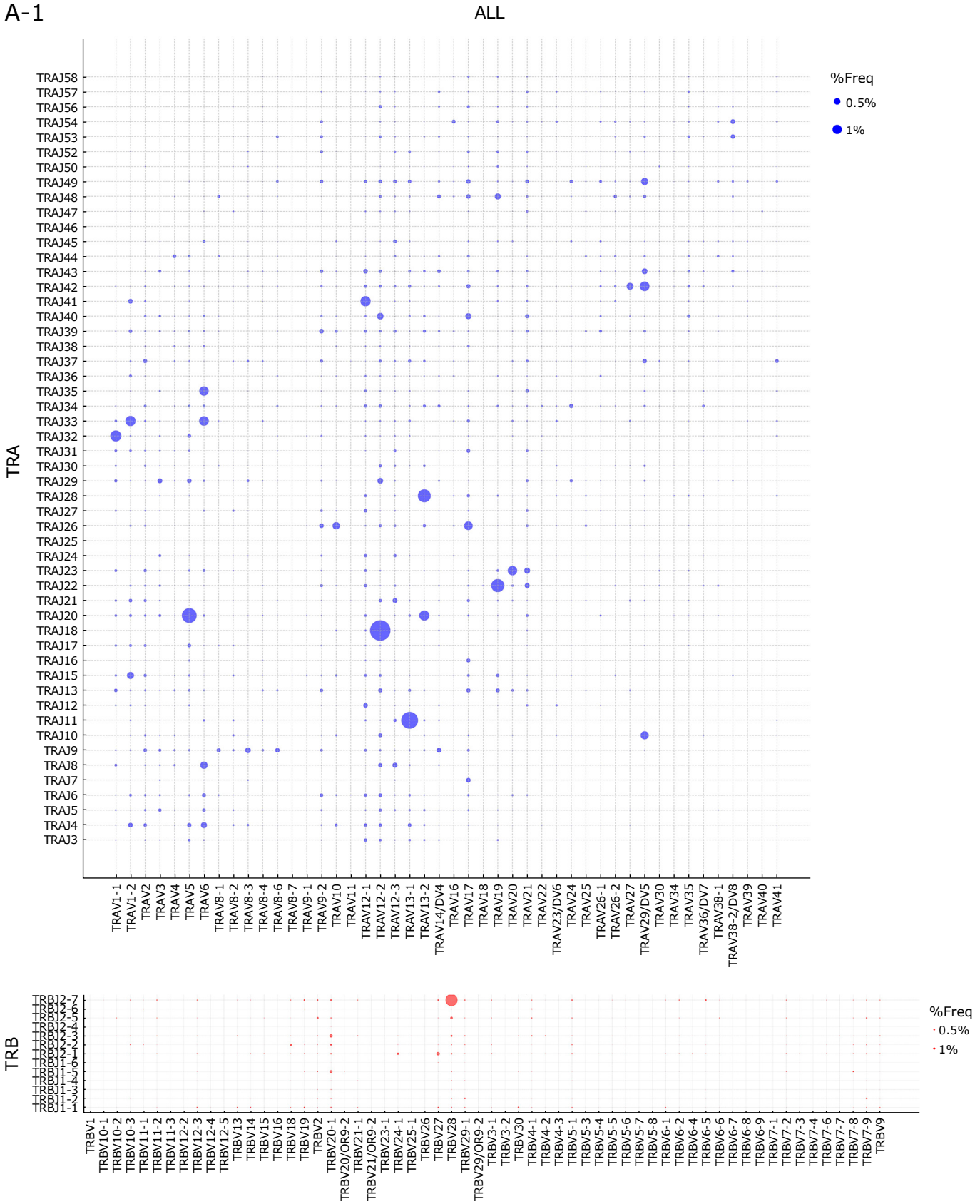
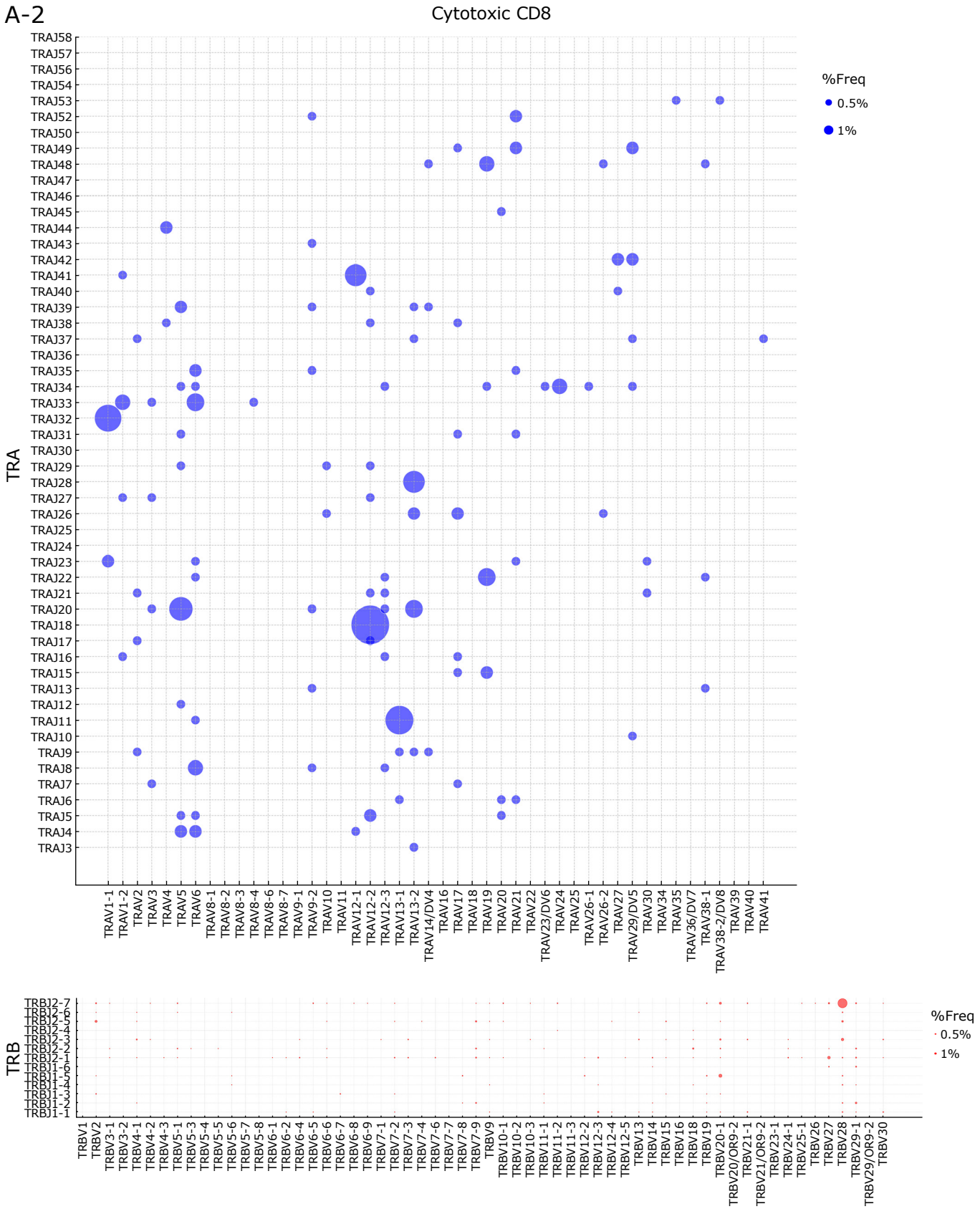


Fig. 6. The diversity of TCR repertoire was reduced by insulin antigen stimulation. (A) Bubble plots showing TCR repertoires of whole cells, cytotoxic CD8⁺ T cells, cytotoxic CD4⁺ T cells, Th1, Th2, Th17, and Treg in all four groups. (C) Shannon index of TCR repertoire and CDR length (amino acids) in T cells. (D) Motif-based sequence analysis tool MEME was used to perform motif analysis common to the GAD, IA-2, and insulin groups in cytotoxic CD8⁺ T cells, cytotoxic CD4⁺ T cells, Th1, Th2, Th17, and Treg in all four groups. Data are presented as mean ± SD values. Data were analyzed using one-way ANOVA with Holm-Šídák's multiple comparison test. *p<0.05.



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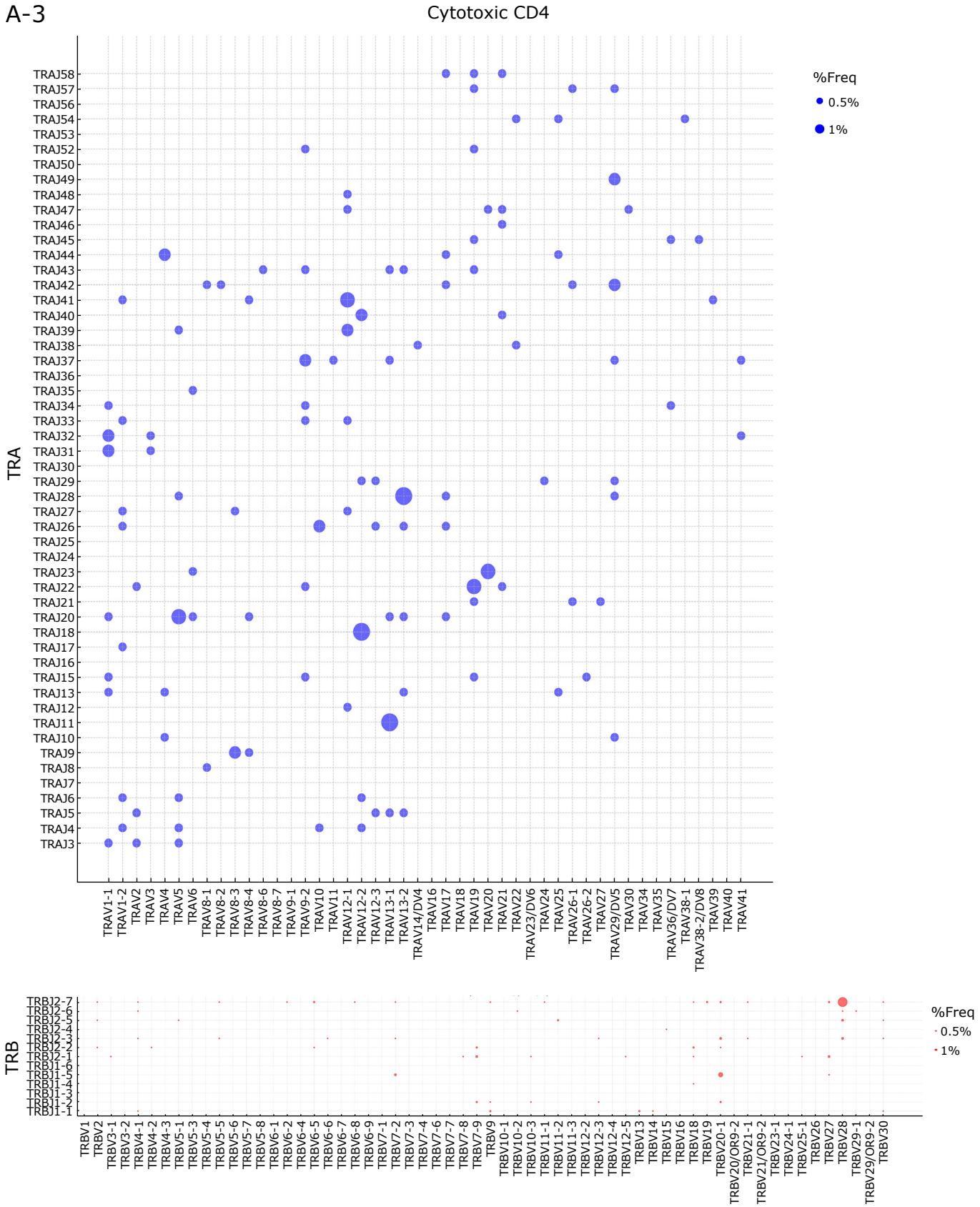

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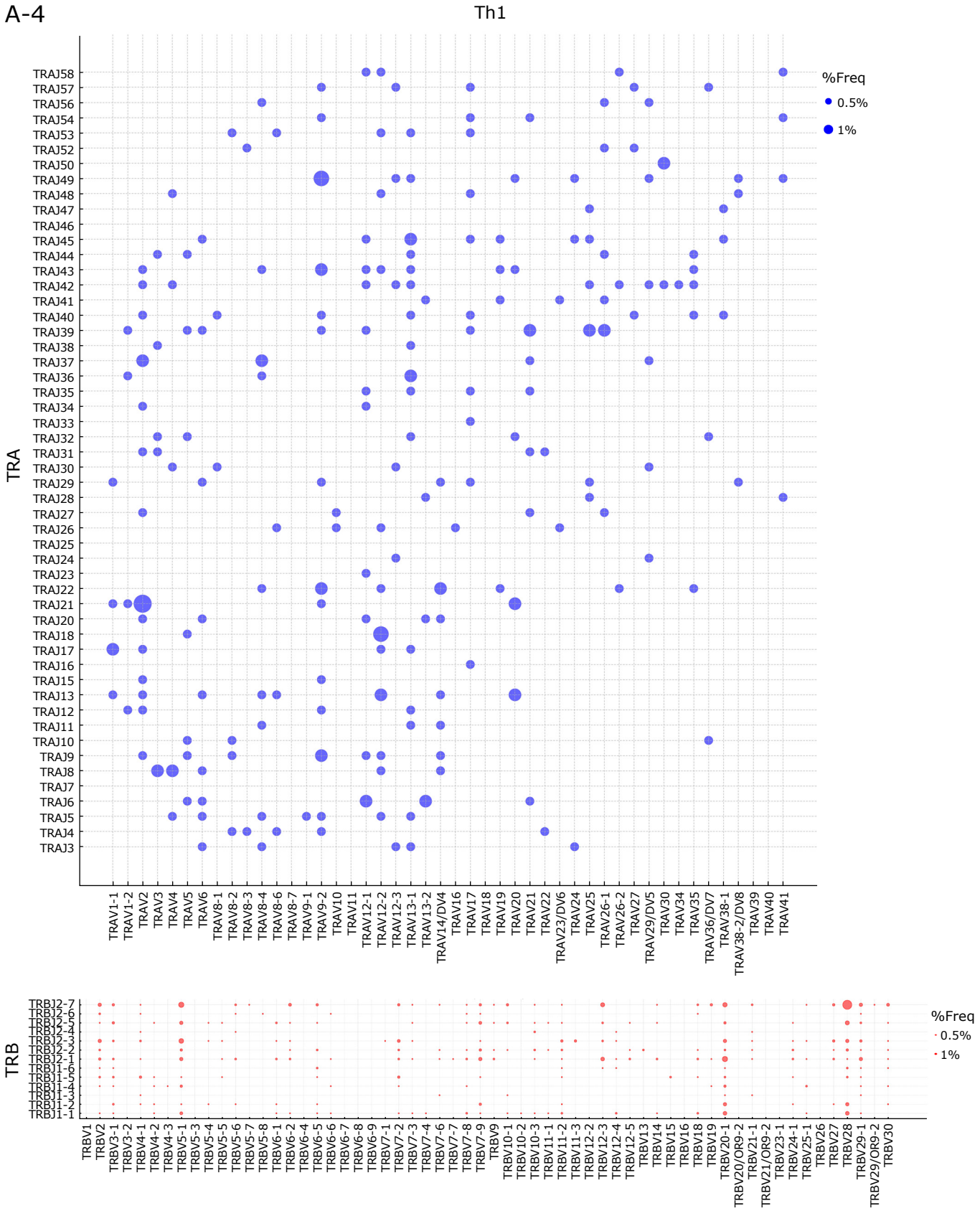

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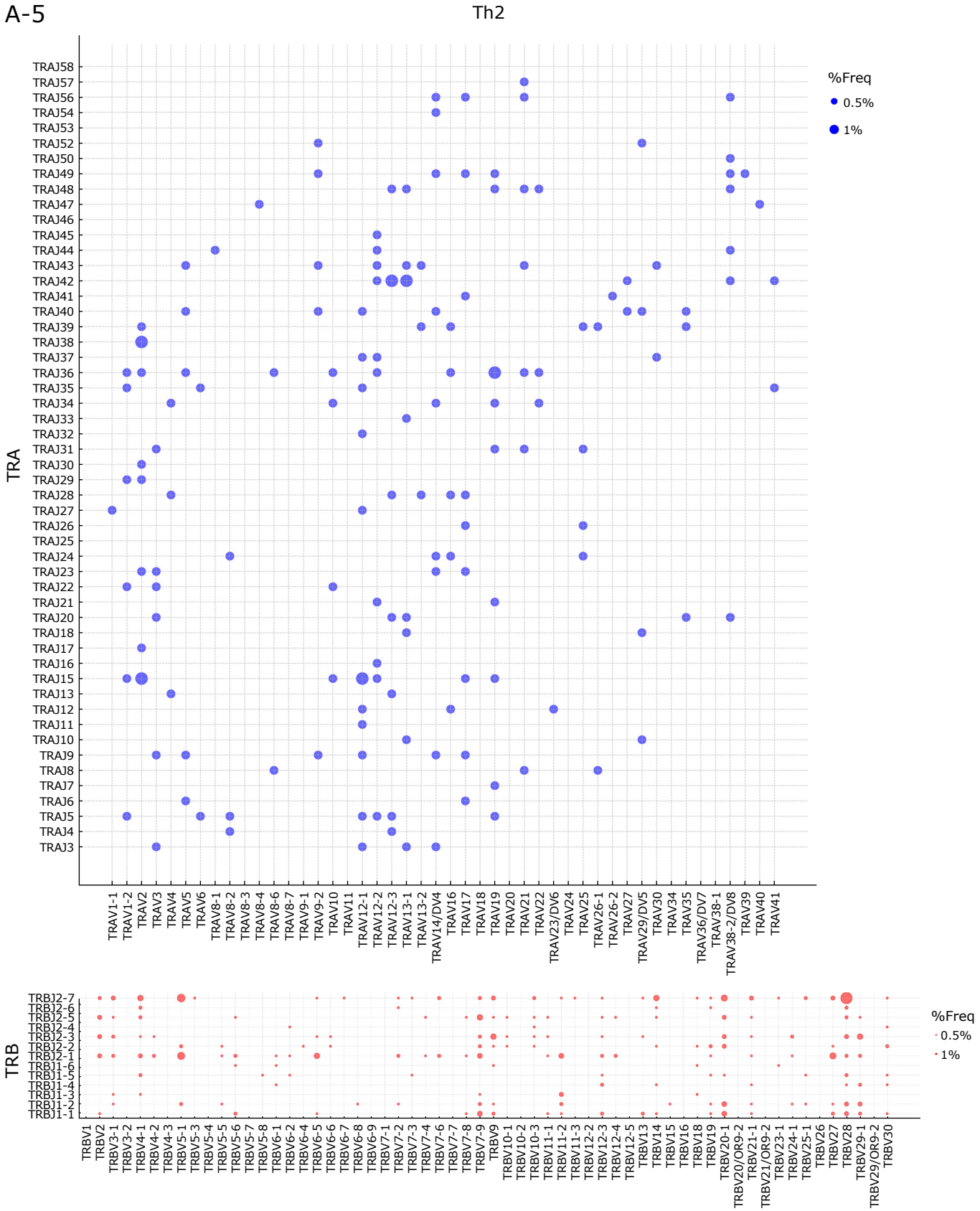

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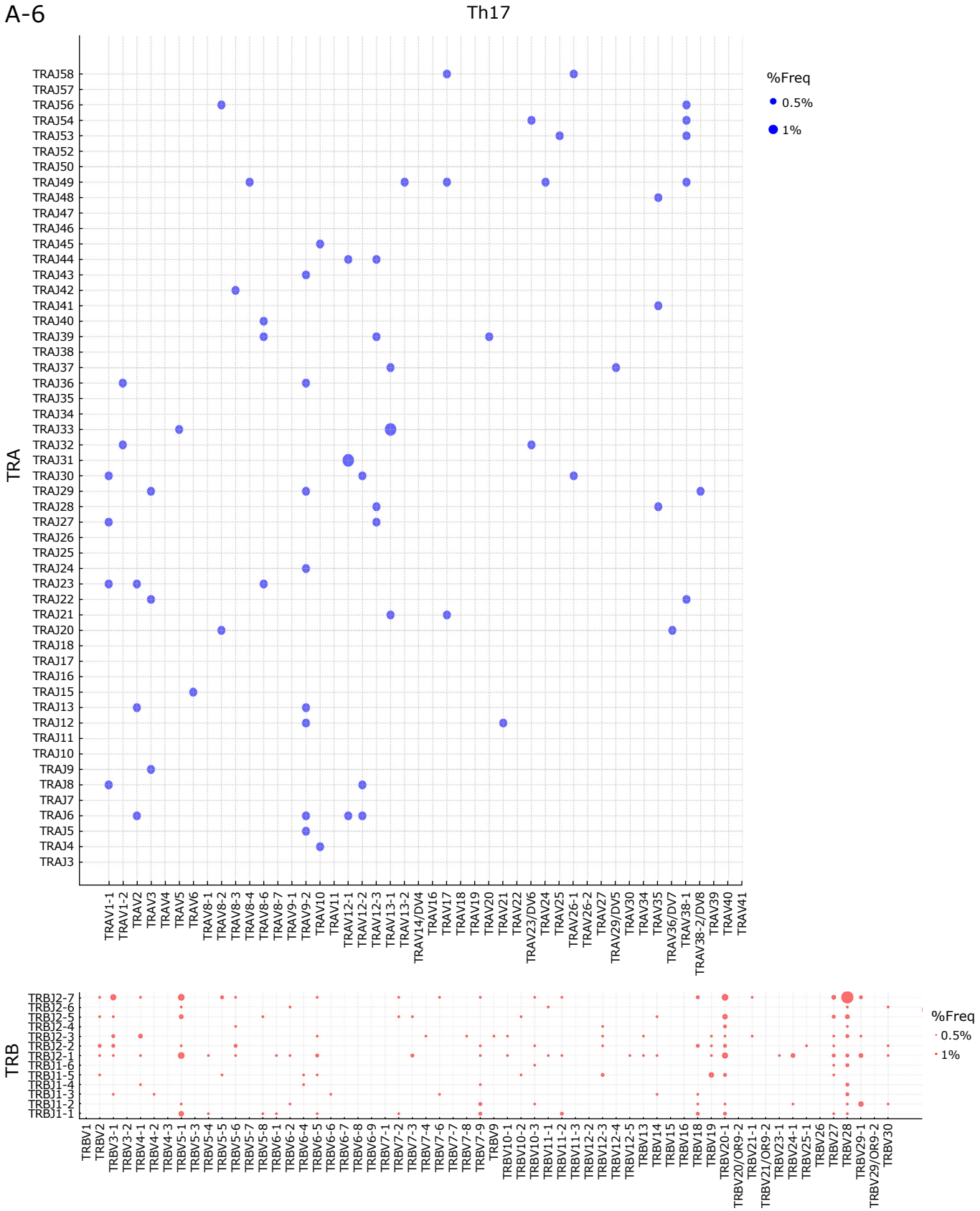

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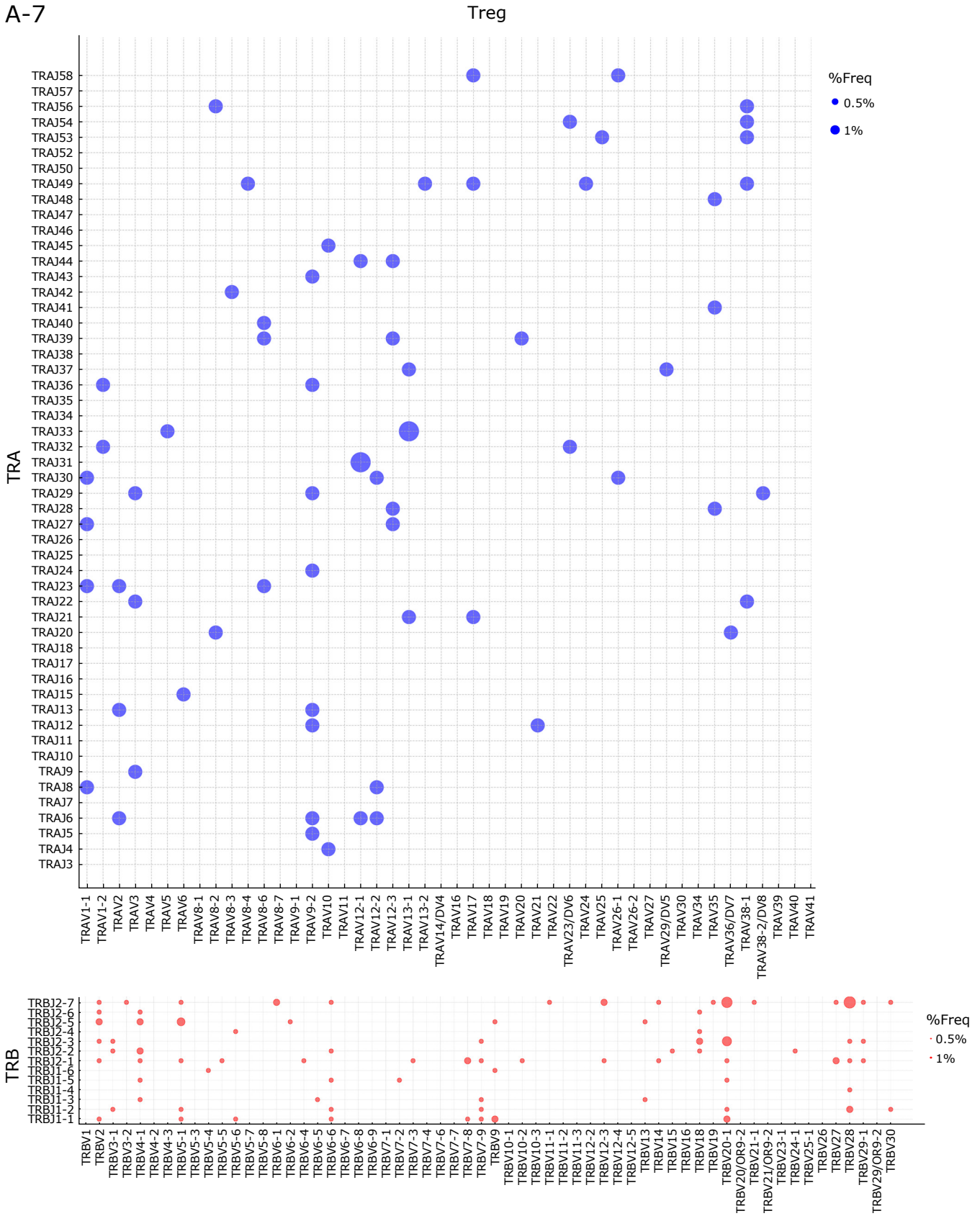

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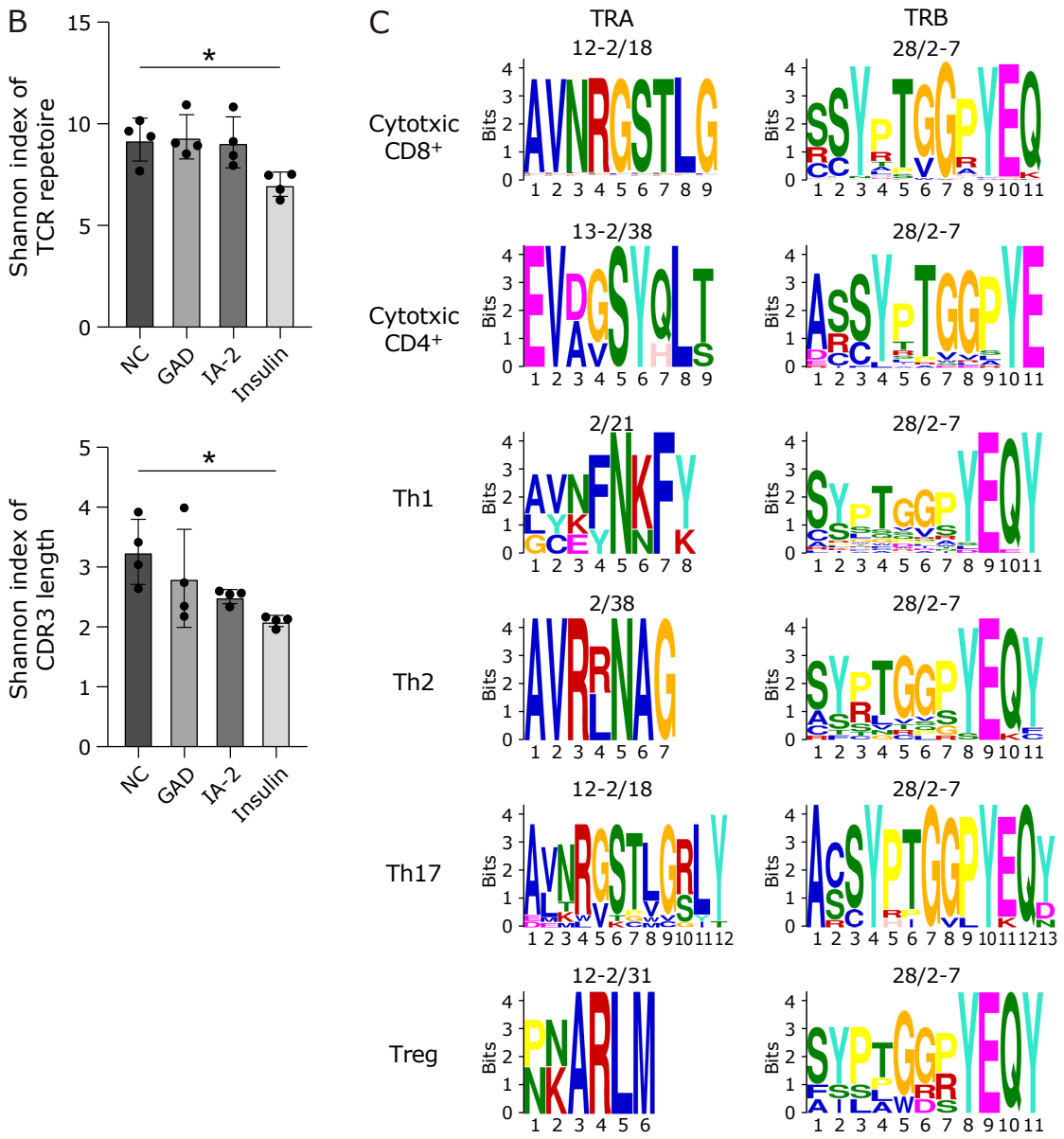

Fig. 6. Continued

ability of HLA-DR$^+$ NK cells to process and present specific antigens on their surface, thus stimulating the activation and proliferation of specific T cells, has been reported.[36] Then, the expression of IL32 was elevated in HLA-DR$^+$ NK cells. there are various reports on the relationship between T1DM and IL-32. mRNA levels of IL-32 in beta cells of type 1 diabetic patients were higher than in control subjects,[56] and IL-32, streptozotocin-induced 1 has been reported to hasten T1DM.[57] The expression of markers indicative of activation such as *GNLY*, *GZMH*, and *GZMK* in dendritic cells and *TNF* and *TGFB1* in B cells were elevated in the insulin group. These results indicate that insulin antigens shift PBMCs to inflammatory, especially in T1DM patients. On the other hand, Th2 had elevated gene expression of *IL4* and *IL13* and Treg had elevated that of *TGFB1* in the insulin group. IL-4 and IL-13 are major effector cytokines produced by Th2 during type 2 immune responses,[58] and Ukah *et al.*[59] found that the IL4/IL-13 double-knockout NOD mice delayed the onset of diabetes by increasing the frequency of mTGFβ + Foxp3int Tregs and the persistence of CD206$^+$ macrophages in the pancreas. Tregs have been reported to inhibit effector T cell activity via TGFβ. Since Th2 and Treg have been reported to be protective against the development of T1DM, this result seemingly contradicts the phenomenon of an inflammatory shift in various immune cells. Although the cause of this phenomenon is not clear, it is expected to be a negative feedback response to the inflammatory shift of various immune cells.

In addition, we have investigated TCR clones and V(D)J recombination. The diversity of TCR clones is known to be reduced in inflammatory conditions, such as in patients with inflammatory bowel disease.[60] In this study, the diversity of TCR was significantly lower in the insulin group than in the other three groups. Interestingly, motif analysis of CDR3 of TCR revealed that TRB is monoclonally increased in the TRBV28/TRBJ2-7 combination. On the other hand, TRAs were relatively scattered among all immune cells, but cytotoxic CD8$^+$ T cells and Th17 showed an increase in the TRAV12-2/TRAJ18 combination, and moreover, their motifs were very similar.

Limitations of this study include the small number of patients

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in whom single-cell sequencing was performed (four patients) and the fact that all patients were more than 10 years from the onset of disease. All patients were already using insulin injections for a long period of time when the PBMCs used in the experiment were collected. This has the potential to bias the various responses to insulin antigen stimulation in this study and, as noted, may allow for a different response when PBMCs are used from untreated patients. Furthermore, the disease type was only type IA, and the human leukocyte antigen (HLA) typing was unknown. These issues are currently being investigated and will be addressed in the future.

In summary, insulin antigen significantly activated immune cells and upregulated inflammatory cytokine expression in PBMCs from type 1 diabetic patients, even when compared to GAD and IA-2 antigens, and promoted a more robust inflammatory shift. Furthermore, this is the first report of TCR sequencing using islet autoantigens. This study provides clues to further elucidate the pathogenesis of T1DM and may serve as a potential therapeutic target for T1DM.

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Abbreviations

ANOVA	analysis of variance
CDR3	complementarity-determining region 3
CCA	canonical correlation analysis
DBEC	distribution-based error correction
FACS	fluorescence-activated cell sorting
GAD	glutamic acid decarboxylase
HLA	human leukocyte antigen
IA-2	islet antigen 2
IFNG	interferon gamma
IL	interleukin
IMGT	international ImMunoGeneTics information system
MHC	major histocompatibility complex
NC	negative control
NK	natural killer
PBMCs	peripheral blood mononuclear cells
scRNA-seq	single-cell RNA sequencing
T1DM	type 1 diabetes mellitus
TCR	T cell receptor
TRAV	T cell receptor alpha variable
TRBJ	T cell receptor beta joining
TRBV	T cell receptor beta variable
Treg	regulatory T cells
TTA	targeted transcriptome analysis
UMAP	uniform manifold approximation and projection
VDJ	variable-diversity-joining
WTA	whole transcriptome analysis

Conflict of Interest

MH received grants from AstraZeneca K.K., Ono Pharma Co. Ltd., Kowa Pharma Co. Ltd., and received personal fees from AstraZeneca K.K., Ono Pharma Co. Ltd., Eli Lilly, Japan, Sumitomo Dainippon Pharma Co., Ltd., Daiichi Sankyo Co. Ltd., Mitsubishi Tanabe Pharma Corp., Sanofi K.K., and Kowa Pharma Co. Ltd. outside of the submitted work.

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